CHAPTER 6

The IL-15 driven downregulation of CD28 T cells is mediated by induction of the miR-23a~24-2 cluster

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Work in progress

Abstract

Accumulation of CD28- cytotoxic T lymphocytes (CTLs) is one of the most prominent feature of T-cell ageing *in vivo*. *Ex-vivo* culture of CD28+ naïve CTLs in the presence of IL-15 also induces loss of CD28. Moreover, CD28- CTLs are characterized by high levels of the members of the miR-23a~24-2 cluster compared to CD28+ CTLs. Here we tested whether the IL-15 driven downregulation of CD28 is mediated through the induction of the three miRNAs of this cluster. We confirmed significant higher levels of all three miRNA cluster members in CD28- as compared to CD28+ CTLs. Treatment of CD28+ CTLs with IL-15 induced downregulation of CD28 and a significant induction of miR-23a, miR-24 and miR-27a. Both the downregulation of CD28 and the induction of these miRNAs were more pronounced in IL-15 treated truly naïve CTLs (CD8+CD28+CD45RO-CCR7+) as compared to CD8+CD28+ CTLs. The induction was most prominent for miR-24 and miR-27a. By luciferase reporter assays we showed that both miR-24 and miR-27a can directly bind to the 3'-UTR of the CD28 transcript. Preliminary data show a mild decrease of CD28 expression upon exogenously expression of miR-24 and miR-27a in Jurkat cells. We confirm IL-15-induced downregulation of CD28 in truly naïve CTLs and show a pronounced IL-15 induced upregulation of the miR-23a~24-2 cluster that possibly contributes to the subsequent downregulation of CD28.

Key words: T cells, microRNA, CD28, IL-15, ageing

Introduction

Loss of CD28 in both naïve and memory CD8+ cytotoxic T lymphocyte (CTL) subsets has been regarded as one of the most profound and consistent age-associated feature of T cells (1-2). CD28-CD8+ T cells are derived from CD28+ as a consequence of active cell proliferation, especially in response to latent viral infections, commonly observed in the elderly population (3-4). Downregulation of CD28 on CD8+ T cells has been well documented not only in response to TCR cross-linking but also in response to homeostatic cytokines, such as those which share common γ-chain receptors including IL-15 (2,5,6). IL-15 is a homeostatic cytokine which supports cell survival and proliferation of naïve CD8+CD28+ cells in the absence of continuous TCR stimulation (2). However, little is known about the mechanisms that underlie the IL-15 mediated loss of CD28.

High expression of the three members of the miR-23a~24-2 cluster has been shown in CD8+CD28- T cells (7). Increased expression of miR-24, i.e one of the member of this cluster, has been reported in CD28- T cells and was associated with an increased susceptibility to cell death, which was counterbalanced by IL-15 (7). Age-related accumulation of the CD8+CD28- T cell population has been reported in elderly individuals and was more pronounced in the bone marrow than in circulation. This accumulation was shown to be driven by a local age-related increase of IL-15 and to a lesser extent by IL-6 (8).

In this study, we tested the hypothesis that the IL-15 induced loss of CD28 on CTLs is mediated by the induction of the miR-23a~24-2 cluster.

Results and Discussion

We first determined the effect of IL-15 on the regulation of the miR-23a~24-2 cluster members in CD8+CD28+ and CD8+CD28- sorted T cells from peripheral blood of 6 healthy young study participants (median age 27) (Supplementary Table S1). We observed a significant induction of miR-24, miR-27a and miR-23a in CD8+CD28- cells when compared to CD8+CD28+ T cells (Supplementary Figure S1 B-D). These findings are in line with the results described in a recent study (7). To assess the effect of IL-15 on the expression of these miRNAs, CD8+CD28+ T cells were cultured and stimulated with different concentrations of IL-15 for 5 days. Expression levels of all three miRNAs were significantly induced in IL-15 stimulated versus non stimulated or IL-4 stimulated cells (Figure 1A-C). This shows that the induction is specific for IL-15 and is not a general activation dependent effect. Induction of miR-23a, miR-24 and miR-27a expression in T cells upon IL-15 stimulation has not been reported previously.

Since it is known that CD28 expression is downregulated in actively proliferating cells in the presence of IL-15, we next assessed CD28 loss. CD3+CD8+CD28+ T cells showed a moderate downregulation of CD28 after stimulation with IL-15 for 15 days (Supplementary

Figure S1 E). The moderate downregulation of CD28 is likely related to the differential sensitivity T-cell subsets present within the CD3+CD8+CD28+ population, i.e. naïve and memory T cells, to the IL-15 induced CD28 downregulation. Indeed, naïve CD8+CD28+ T cells have been shown to strongly respond to IL-15 with a marked downregulation of CD28 (2).

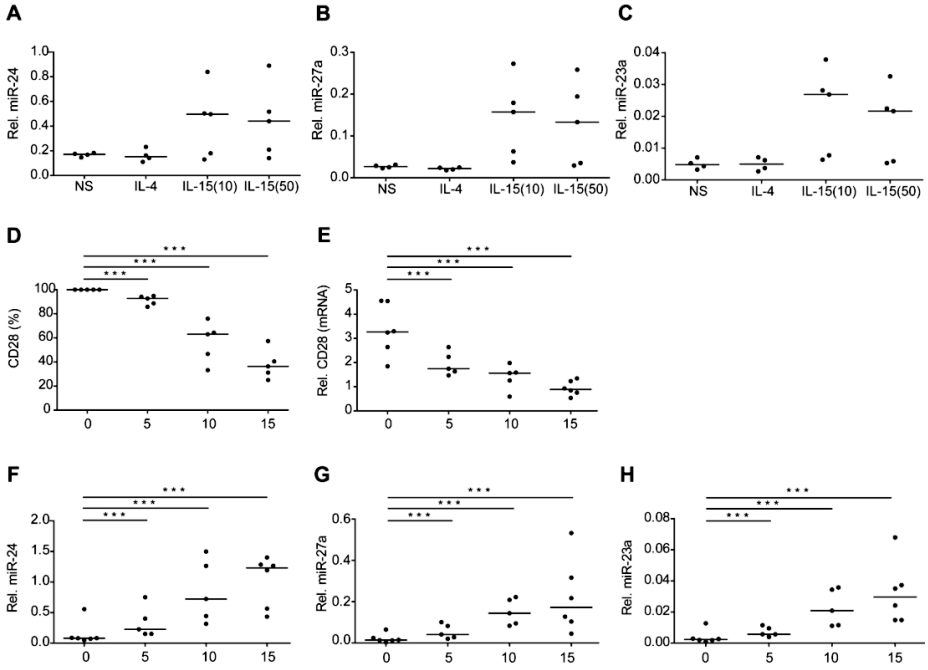

Figure 1. IL-15 induces expression of members of the miR-23a~24-2 cluster and downregulates expression of CD28 in naïve CD8+ T cells. A, Expression levels of miR-24 **B,** miR-27a **C,** miR-23a in CD3+CD8+CD28+ T cells stimulated with IL-4 or IL-15 and in untreated cells. **D,** Dynamics of CD28 loss in naïve CD3+CD8+ CD45RO-CCR7+CD28+ T cells at the protein and **E,** mRNA level following IL-15 stimulation for 15 days. **F,** expression level of miR-24, **G,** miR-27a and **H.** miR-23a in naïve CD3+CD8+ CD45RO-CCR7+CD28+ T cells following IL-15 stimulation for 15 days. Expression of miRNAs was normalized to the expression of RNU48 (lines indicate median), expression of CD28 mRNA was normalized to expression of TBP (lines indicate median) ***p ≤ 0.001.

To study the dynamics of CD28 expression as well as expression of the miR-23a~24-2 cluster members upon IL-15 stimulation, we next sorted naïve CD8+CD28+ T cells from 6 healthy young donors (median age 28, Supplementary Table S1 and Figure S2 A). Expression of CD28 protein and mRNA levels significantly reduced after 15 days of stimulation with IL-15. The percentage of CD28+ T cells decreased from 100% at day 0 to 36% at day 10 and this loss was confirmed at the mRNA level (Figure 1D,E). Expression of miR-23a, miR-27a and miR-24-2 significantly increased upon IL-15 stimulation (Figure 1F-

H). IL-15 treated naïve CD8+CD28+ cells gained a memory phenotype with enhanced expression of CD45RO and decreased expression of CCR7 (Supplementary Figure S2 B,C) consistent with previous studies (2). In addition, the majority of the cells gained expression of the cellular senescence marker, p16INK4a (Supplementary Figure S2 D). These findings indicate that the IL-15 mediated induction of the miR-23a~24-2 cluster is associated with downregulation of CD28 and upregulation of p16INK4a.

Several miRNA target gene prediction algorithms denote two conserved miR-24 and miR-27a and one conserved miR-23a binding site in the 3'UTR of CD28. This suggests a direct relation between the loss of CD28 and the induction of the miR-23a~24-2 cluster members (Figure 2A).

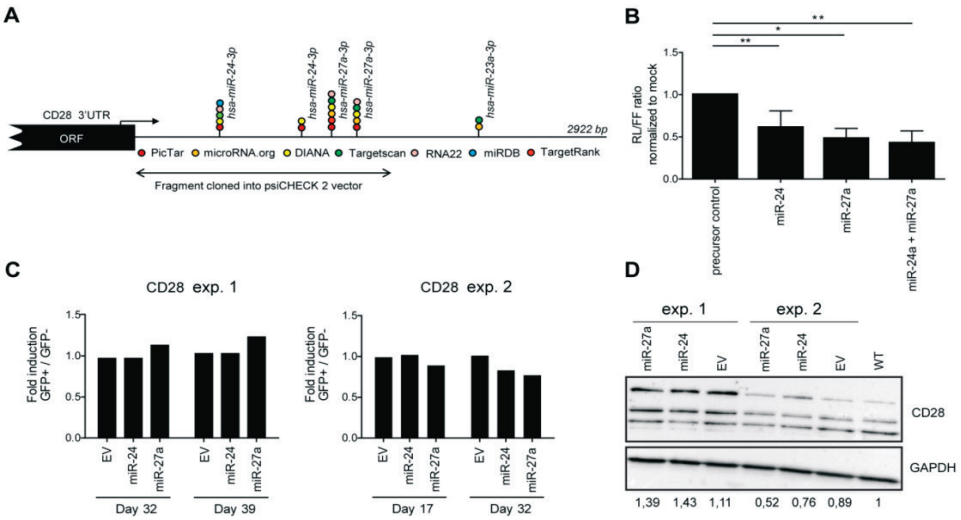

Figure 2. CD28 is a direct target of miR-24 and miR-27a. A, Schematic overview of miR-24, miR-27a and miR-23a binding sites in the 3'UTR of the CD28 transcript. **B,** Results of the luciferase reporter assay of miR-24 and miR-27a alone or combined shows that these miRNAs can interact with the 3'UTR of CD28. Cos-7 cells were co-transfected with psiCHECK-2 construct harboring the CD28 3'UTR and control miR-24, miR-27a or a combination of miR-24 and miR-27a miRNA precursors. Shown is the Renilla over Firefly luciferase ratio set to 1 for the control precursor transfected cells. **C,** Quantification of CD28 surface expression in Jurkat T cells upon miR-24 or miR-27a overexpression. Two independent experiments are shown (Exp. 1 and Exp. 2). Mean fluorescence intensity values (MFI) were determined in EV, miR-24 and miR-27a overexpressing cells. Shown are the fold changes calculated from MFI values of the transduced (GFP+) cells normalized to non-transduced (GFP-) cells. **D,** Quantification of CD28 expression in Jurkat T cells by Western blot. Cells were harvested from the same 2 independent experiments as shown in C. For exp.1 cells were harvested at day 39 and for exp.2 at day 32. GAPDH was used a loading control. *p ≤ 0.05, **p ≤ 0.01.

To determine if these miRNAs indeed bind to the 3'UTR of the CD28 transcript, we performed a luciferase reporter assay in COS-7 cells. As miR-23a expression levels were

very low, we focused our analysis on miR-24 and miR-27a. Significant reduction in relative luciferase levels were observed both for miR-24 and for miR-27a co-transfected cells and not in control transfected cells (Figure 2B). This confirms that miR-24 and miR-27a directly target the 3'UTR of the CD28 transcript.

To determine the effect of miR-24 and miR-27a on regulation of CD28 expression in Jurkat T cells, we stably overexpressed miR-24 and miR-27a in Jurkat T cells using a viral vector system (Supplementary Figure S3). Initially, we did not observe an effect of miRNA overexpression on the MFI of CD28 at day 32 (Figure 2C (exp. 1)) However, when we repeated the experiment we observed a slight decrease of the MFI of CD28 at day 17 and 32 post infection with the miR-27a vector in GFP+ sorted Jurkat T cells (Figure 2C (exp. 2)). For miR-24 we only observed an effect at day 32. The flow cytometry results of experiment 1 and 2 were confirmed by Western blot, with no effect for experiment 1 and a slight reduction in CD28 levels in experiment 2 (Figure 2D). Further experiments are ongoing to establish a potential effect of these miRNA on loss of CD28 expression in Jurkat and primary T cells.

In summary, our results revealed a clear IL-15 induced expression of miR-24 and miR-27a in naïve CTLs. Luciferase results support a direct role of miR-24 and miR-27a in the regulation of CD28 expression, but this needs to be confirmed at the protein level. IL-15 driven downregulation of CD28 has in part been attributed to the induction of TNFα secretion (6), which alters the promoter activity of the CD28 gene (5). However, we did not study a possible role of TNFα on the miRNAs or on CD28 in this study. Thus, in Jurkat cells, miR-24 and miR-27a in combination with additional CD28 regulatory factors, such as TNFα, regulate expression of CD28. Therefore, it would be of interest to study IL-15 mediated CD28 loss in a TNFα deficient condition.

Conclusion

Taken together, we show preliminary results indicating the involvement of IL-15 induced expression of miR-24 and miR-27 in the regulation of CD28 loss in naïve CTLs.

Acknowledgements

We are grateful to all healthy young volunteers for participating in the study. We thank the flow cytometry team, Geert Mesander, Henk Moes and Roelof Jan van der Lei for the excellent support.

Materials and Methods

Participants

28 healthy young (≤ 30 yrs) individuals were included in this study. Demographic characteristics of all donors are summarized in Supplementary Table S1. Informed consent was obtained of all participants in accordance with the Declaration of Helsinki. The Medical Ethical Committee (METC) of the University Medical Center Groningen approved the study.

Fluorescence-activated cell sorting (FACS) of human primary lymphocyte subsets and analysis of cell surface markers

Peripheral blood mononuclear cells (PBMC) were freshly isolated by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's protocol. CD3+CD8+CD28+, CD3+CD8+CD28- and CD3+CD8+CD45RO-CCR7+CD28+ T cells were isolated by fluorescence-activated cell sorting (FACS) using combinations of the following anti-human monoclonal antibodies: anti-CD3-e450, anti-CD8a-APC-e780 (eBioscience, Vienna, Austria), anti-CD45RO-FITC, anti-CCR7-PE (BD Biosciences, Breda, The Netherlands), anti-CD28 PeCy7 (Biolegend, Uithoorn, The Netherlands). Sorting strategies are shown in Supplementary Figures S1A and S2A. Cells were sorted using a MoFlo flow cytometry cell sorter (Beckman Coulter, Woerden, The Netherlands).

Expression of cell surface markers on T cells was assessed using mAbs against human CD28-PECY7 (CD28.2) (Biolegend), CCR7-PE (3D12) and CD45RO-FITC (UCHL1) (BD Biosciences). Cells were analyzed using a BD LSR-II Flow Cytometer and the Diva software (BD Biosciences). Data analysis was done using the Kaluza Flow Analysis Software (1.2) (Beckman Coulter).

RNA extraction and purification

Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. Micro Bio-Spin^{TM} chromatography columns, supplied with Bio-Gel P-6 polyacrylamide gel matrices, were applied to purify the obtained RNA samples (Bio-Rad laboratories B.V. Veenendaal, The Netherlands). RNA concentration was measured on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). RNA samples with 260/280 and 260/230 ratio of ≥ 1.90 were used for further analysis.

Quantitative-RT-PCR

MiRNA expression levels were determined by quantitative RT-PCR. Multiplexed cDNA synthesis for all miRNAs and the reference gene was performed using Taqman MicroRNA Reverse transcription kit and reverse transcription primers of TaqMan microRNA Assays (Life Technologies, Amsterdam, the Netherlands) as described earlier (9). Assays used: miR-23a (000399), miR-24 (000402), miR-27a (000408) and RNU48 (001006). RNU48 served as a reference gene to normalize miRNA expression levels.

cDNA synthesis for mRNA was performed using Superscript III RTase (Life Technologies). The qPCR reaction was performed using qPCR MasterMix Plus (Eurogentec, Liege, Belgium). Primers and probe (Integrated DNA Technologies, Coralville, USA) used for detection of TBP: forward 5'-GCCCGAAACGCCGAATAT-3', reverse 5'-CCGTGGTTCGTGGCTCTCT-3', probe 5'-6-FAM-ATCCCAAGCGGTTTGCTGCGG-BHQ-1-3' and p16INK4a: forward 5'-CCAACGCACCGAATAGTTACG-3', reverse 5'GCGCTGCCCATCATCATG-3', probe 5'-FAM-CCTGGATCGGCCTCCGAC-MGB-3'. TBP served as a reference gene to normalize CD28 mRNA and p16INK4a expression levels. Taqman gene expression assay was used for detection of CD28: Hs01007422_m1 (Life Technologies).

All PCR reactions were run in triplicate. Mean cycle threshold (Ct) values were quantified with the Sequence Detection Software (SDS, version 2.3, Life Technologies). Relative expression levels were shown as $2^{-\Delta Ct}$ (ΔC_t= C_t gene – C_t reference gene) values.

T cell stimulation with human recombinant IL-15

FACS sorted CD3+CD8+CD28+ and CD3+CD8+CD28+CD45RO-CCR7+ T cells were suspended in RPMI medium (Lonza, Breda, The Netherlands) supplemented with 10mg/ml gentamycin sulfate (Lonza) and 10% fetal bovine serum (FBS)(Thermo Scientific, Breda, The Netherlands) in a volume of 3mL and seeded at a density of $1x10^6$/mL in T25cm flasks. A final concentration of 50ng/mL human recombinant IL-15 (Peprotech, London, UK) or 20ng/mL human recombinant IL-4 (R&D, Minneapolis, USA) was added to the cell culture at day 0 and refreshed every 5th day. T cells were cultured in a humidified atmosphere at 37ºC in 5% CO2. On day 5, 10 and 15 of culture, cells were harvested and stained for flow analysis and / or lysed for RNA isolation.

Culture of COS-7 cells

COS-7 cells (African Green Monkey SV40- transformed kidney fibroblast cell line) were cultured in Dulbecco modified Eagle medium (DMEM)(Lonza) supplemented with 10% fetal bovine serum (Thermo Scientific), 200mM L-glutamine and 10mg/mL gentamycin sulfate (Lonza,) in a humidified atmosphere at 37^0C in 5% CO_2.

Cloning of 3'UTR in a luciferase reporter construct, transient transfection and luciferase reporter assays

The CD28 3'UTR sequence harboring the putative miR-24, miR-27a and miR-23a binding sites was amplified from genomic DNA using primers containing an XhoI (5') or NotI (3') restriction site, forward: 5'- GCUCCUGCACAGUGACUACA-3', reverse 5'-ACCUUCUGCCUGACCACUUC-3' and cloned into psiCHECK2 vector (Promega, Leiden, the Netherlands), as previously described (10). The insert was sequence verified (BaseClear, Leiden, The Netherlands). COS-7 cells (1,2 x10^4) were transfected with 125ng of the psiCHECK2 construct and either 50nM hsa-miR-24 (MC10737), hsa-miR-27a (MC10939) mimics or a miRNA precursor negative control #1 (Life Technologies) using the Saint-MIX compound (Synvolux Therapeutics BV. Groningen, Netherlands) according to the manufacturer's protocol. Cells were lysed 48hrs after transfection and Renilla (RL) and Firefly (FF) luciferase activity was assessed using Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's protocol. For each transfection, luciferase activity was measured in duplicate with the Luminoskan Ascent Microplate Luminometer (Thermo Scientific). The RL/FF) luciferase ratio for miR-24 and miR-27a was calculated. The RL/FF ratio of control precursor was set to one.

Viral transduction

Lentiviral particles were produced by calcium phosphate mediated transfection of HEK293T cells with 2,5M CaCl2, 1µg pMSCV-VSV-G, 1µg pRSV.REV, 1µg pMDL-gPRRE and 2µg pCDH-EF1-MCS-IRES-GFP. Empty vector and miR-24 and miR-27a overexpression constructs were obtained from SBI (System Biosciences, Uden, The Netherlands). Vector without insert was used as a negative control (EV). Lentiviral particles were passed through a 0,45µM millex-HV PVDF filter (Millipore, Amsterdam, The Netherlands) before infection. Jurkat cells at a concentration of 250,000 cells/mL were transduced with the virus and placed in a 6 wells plate in a humidified atmosphere at 37^{0}C and 5% CO2 overnight. The next day, cells were washed 3x times with PBS and diluted to 250,000 cells/mL. Transduced cells were sorted based on expression of green fluorescent protein (GFP) using a MoFlo flow cytometry cell sorter (Beckman Coulter).

Western blotting

Cells (5x10^6 cells) were lysed in 250µL Radio ImmunoPrecipitation Assay (RIPA) lysis buffer (Millipore). Protein concentration was measured with the Pierce™ BCA Protein Assay Kit (Thermo Scientific). 15 µg of total protein was run on 12,5% polyacrylamide gel and transferred to nitrocellulose blot. The blot was blocked with 5% milk in Tris buffer saline tween (TBST) for 1hr and incubated overnight with a polyclonal goat antibody against CD28,

(1:500, ab113358, Abcam, Cambridge, UK) and monoclonal antibody against GAPDH (1:20,000, SC47724, Santa Cruz, Heidelberg, Germany) in 5% milk in Tris buffer saline tween (TBST) at room temperature for 1hr. After being rinsed, the blot was incubated with peroxidase-labeled secondary Rabbit anti-mouse antibody (1:1000 dilution) for 1hr for the detection of GAPDH and with Rabbit anti-goat (1:1000) for the detection of CD28. Staining was visualized using Supersignal ® Chemiluminescent Substrate (Thermo Scientific). Band intensities were quantified using the Gel Doc^{TM} EZ system software. (Bio-Rad).

Statistical analysis

Results obtained from qRT-PCR are expressed as median ± standard deviation respectively. Differences between paired samples were tested using the Wilcoxon signed-rank test. For comparisons of paired unstimulated vs stimulated samples with unequal replicates at different time points, the generalized estimating equation (GEE) analysis with Wald Chi-Square test was applied. Statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS Statistics version 22.0 (IBM Corp. Armonk, NY, USA). $P<0.05$ was considered statistically significant.

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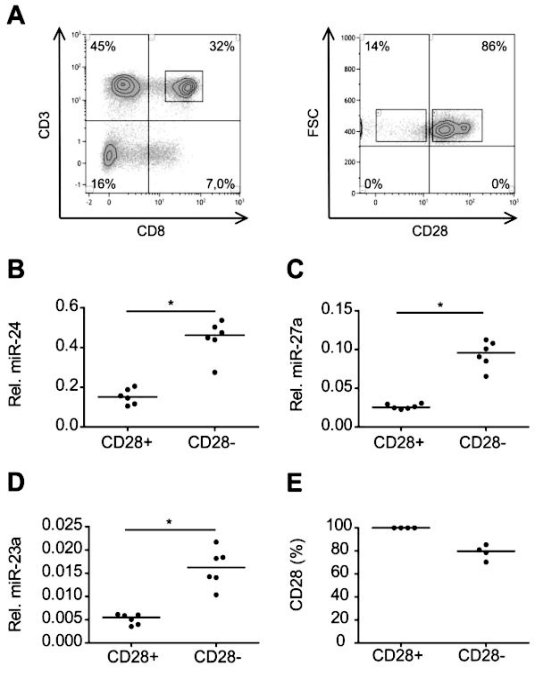
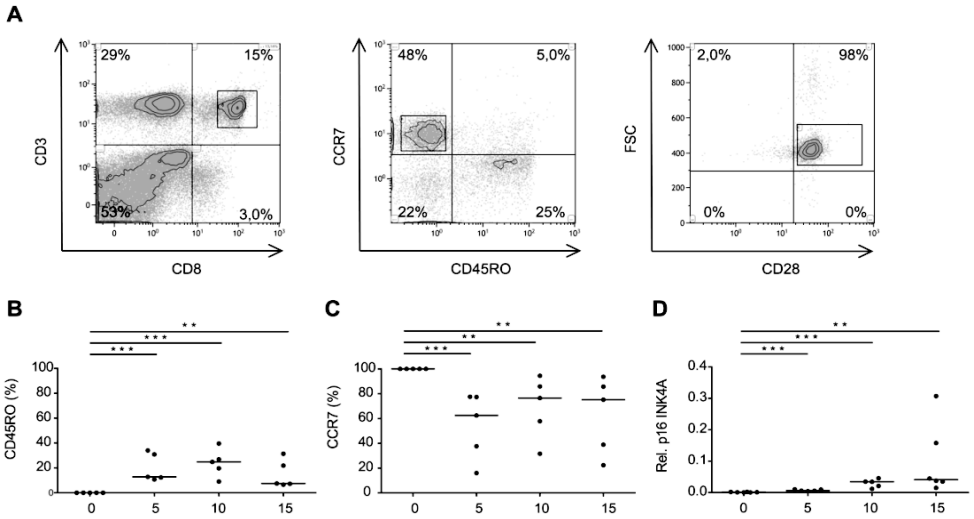
Supporting information

Supplementary Table S1. Characteristics of the study participants and overview of experimentation.

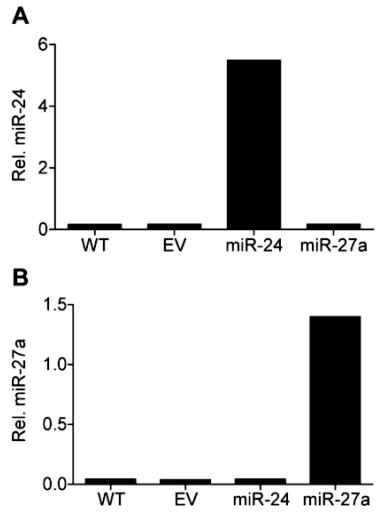
Donors	*Age (y)*	*Sex*	*Isolated T cells*	*Stimulation*	*Analysis miRNA*	*Analysis FACS*
1	34	M	CD3+CD8+CD28+	5 (d)	qRT-PCR	CD28
2	25	M	CD3+CD8+CD28+	5 (d)	qRT-PCR	CD28
3	28	F	CD3+CD8+CD28+	5 (d)	qRT-PCR	CD28
4	24	M	CD3+CD8+CD28+	5 (d)	qRT-PCR	CD28
5	30	M	CD3+CD8+CD28+	5 (d)	qRT-PCR	-
6	28	F	CD3+CD8+CD28+	5 (d)	qRT-PCR	-
7	27	F	CD3+CD8+CD45RO-CD28+	15 (d)	qRT-PCR	CD28
8	25	F	CD3+CD8+CD45RO-CD28+	15 (d)	qRT-PCR	CD28
9	30	F	CD3+CD8+CD45RO-CD28+	15 (d)	qRT-PCR	CD28
10	23	M	CD3+CD8+CD45RO-CD28+	15 (d)	qRT-PCR	CD28
11	28	F	CD3+CD8+CD45RO-CD28+	15 (d)	qRT-PCR	CD28
12	29	F	CD3+CD8+CD45RO-CD28+	15 (d)	qRT-PCR	CD28



Supplementary Figure S1. MiR-24, miR-27a and miR-23a are upregulated in CD8+CD28- vs CD8CD28+ T cells; IL-15 induces mild downregulation of CD28. **A,** Representative example of the sorting strategy of CD3+CD8+CD28+ and CD3+CD8+CD28- T cells. **B,** Expression of miR-24, **C,** miR-27a and **D,** miR-23a in sorted CD8+CD28+ and CD8+CD28- T cells. Expression of miRNAs was normalized to the expression of RNU48 (lines indicate median) **E,** Percentage of CD28 positive cells following stimulation of CD8+CD28+ T cells with IL-15 after 15 days determined by flow cytometer. Cells have been stimulated with IL-15 (50ng/ml). * $p \leq 0.05$.



Supplementary Figure S2. Phenotype of CD3+CD8+CD28+ T cells following treatment with IL-15. **A,** Sorting strategy of naïve CD3+CD8+CD28+CD45RO-CCR7+ T cells. **B,** Percentage of CD3+CD8+CD28+CD45RO-CCR7+ T cells expressing CD45RO and **C,** CCR7 after IL-15 treatment. Expression of cell surface markers was determined by flow cytometry and depicted as percentage of positive cells. **D,** p16INK4A mRNA levels in naïve CD3+CD8+CD28+CD45RO-CCR7+ T cells treated with IL-15. P16INK4A mRNA expression was normalized to the expression of TBP (median is indicated). **p ≤ 0,01, ***p ≤ 0,001.



Supplementary Figure S3. Overexpression of miRNA's via viral vector system. Expression of **A,** miR-24 and **B,** miR-27a in Jurkat cells transduced with virus carrying miR-24 and miR-27a overexpression vectors. MiRNA expression was normalized to the expression of RNU48.